

In re Application of:
Jay M. Short
Application No.: 09/529,458
Filed: May 23, 2000
Page 2

PATENT
Attorney Docket No.: DIVER1380-1

demand for antibacterial drugs in emerging nations with young populations, countries with aging populations, such as the US, require a growing repertoire of drugs against cancer, diabetes, arthritis and other debilitating conditions. The death rate from infectious diseases has increased 58% between 1980 and 1992 and it has been estimated that the emergence of antibiotic resistant microbes has added in excess of \$30 billion annually to the cost of health care in the US alone. As a response to this trend, pharmaceutical companies have significantly increased their screening of microbial diversity for compounds with unique activities or specificities.

Please replace the original paragraph at page 2, lines 12-27 with the following paragraph:

The majority of bioactive compounds currently in use are derived from soil microorganisms. Many microbes inhabiting soils and other complex ecological communities produce a variety of compounds that increase their ability to survive and proliferate. These compounds are generally thought to be nonessential for growth of the organism and are synthesized with the aid of genes involved in intermediary metabolism hence their name -- secondary metabolites. Secondary metabolites that influence the growth or survival of other organisms are known as bioactive compounds and serve as key components of the chemical defense arsenal of both micro- and macroorganisms. Humans have exploited these compounds for use as antibiotics, antiinfectives and other bioactive compounds with activity against a broad range of prokaryotic and eukaryotic pathogens. Approximately 6,000 bioactive compounds of microbial origin have been characterized, with more than 60% produced by the gram positive soil bacteria of the genus *Streptomyces*. Of these, at least 70 are currently used for biomedical and agricultural applications. The largest class of bioactive compounds, the polyketides, include a broad range of antibiotics, immunosuppressants and anticancer agents which together account for sales of over \$5 billion per year.

Please replace the original paragraph at page 2, line 28 to page 3, line 10 with the following paragraph:

In re Application of:

Jay M. Short

Application No.: 09/529,458

Filed: May 23, 2000

Page 3

PATENT

Attorney Docket No.: DIVER1380-1

Despite the seemingly large number of available bioactive compounds, it is clear that one of the greatest challenges facing modern biomedical science is the proliferation of antibiotic resistant pathogens. Because of their short generation time and ability to readily exchange genetic information, pathogenic microbes have rapidly evolved and disseminated resistance mechanisms against virtually all classes of antibiotic compounds. For example, there are virulent strains of the human pathogens *Staphylococcus* and *Streptococcus* that can now be treated with but a single antibiotic, vancomycin, and resistance to this compound will require only the transfer of a single gene, *vanA*, from resistant *Enterococcus* species for this to occur. When this crucial need for novel antibacterial compounds is superimposed on the growing demand for enzyme inhibitors, immunosuppressants and anti-cancer agents, it becomes readily apparent why pharmaceutical companies have stepped up their screening of microbial diversity for bioactive compounds with novel properties.

Please replace the original paragraph at page 3, lines 11-24 with the following paragraph:

The approach currently used to screen microbes for new bioactive compounds has been largely unchanged since the inception of the field. New isolates of bacteria, particularly gram positive strains from soil environments, are collected and their metabolites tested for pharmacological activity. A more recent approach has been to use recombinant techniques to synthesize hybrid antibiotic pathways by combining gene subunits from previously characterized pathways. This approach, called combinatorial biosynthesis has focused primarily on the polyketide antibiotics and has resulted in a number of structurally unique compounds which have displayed activity. However, compounds with novel antibiotic activities have not yet been reported, an observation that may be due to the fact that the pathway subunits are derived from those genes encoding previously characterized compounds. Dramatic success in using recombinant approaches to small molecule synthesis has been recently reported in the engineering of biosynthetic pathways to increase the production of desirable antibiotics.

Please replace the original paragraph at page 11, lines 17-20 with the following paragraph:

In re Application of:

Jay M. Short

Application No.: 09/529,458

Filed: May 23, 2000

Page 4

PATENT

Attorney Docket No.: DIVER1380-1

In addition to protein-protein interactions, the study of the interaction of other molecules, and the ability to affect this interaction, is of interest in research and discovery processes and in the discovery of new drugs, for instance, steroids and their receptors, or polysaccharides and their receptors.

Please replace the original paragraph at page 12, lines 15-21 with the following paragraph:

In the present invention, for example, gene libraries generated from one or more uncultivated microorganisms are screened for an activity of interest. Potential gene pathways encoding bioactive molecules of interest are first captured in prokaryotic cells in the form of gene expression libraries and screened for activities of interest utilizing the methods of the present invention. Screening hosts can be modified to contain proteins or other molecules from metabolically rich cell lines which can aid in the expression of bioactive compounds such as small molecules.

Please replace the original paragraph at page 16, lines 5-11 with the following paragraph:

The microorganisms from which the libraries may be prepared include prokaryotic microorganisms, such as Eubacteria and Archaea, and lower eukaryotic microorganisms such as fungi, some algae and protozoa. Libraries may be produced from environmental samples in which case DNA may be recovered without culturing of an organism or the DNA may be recovered from one or more cultured organisms. Such microorganisms may be extremophiles, such as hyperthermophiles, thermophiles, psychrophiles, psychrotrophs, halophiles, acidophiles, and the like.

Please replace the original paragraph at page 24, lines 7-14 with the following paragraph:

Since it appears that many bioactive compounds of bacterial origin are encoded in contiguous multigene pathways varying from 15 to 100 kbp, cloning large genome fragments is preferred with the present invention, in order to express novel pathways from natural assemblages of microorganisms. Capturing and replicating DNA fragments of 40 to 100 kbp in

In re Application of:

Jay M. Short

Application No.: 09/529,458

Filed: May 23, 2000

Page 5

PATENT

Attorney Docket No.: DIVER1380-1

surrogate hosts such as *E. coli*, *Bacillus* or *Streptomyces* is in effect propagating uncultivated microbes, albeit in the form of large DNA fragments each representing from 2 to 5% of a typical eubacterial genome.

Please replace the original paragraph at page 24, lines 14-30 with the following paragraph:

Two hurdles that must be overcome to successfully capture large genome fragments from naturally occurring microbes and to express multigene pathways from subsequent clones are 1) the low cloning efficiency of environmental DNA and 2) the inherent instability of large clones. To overcome these hurdles, high quality large molecular weight DNA is extracted directly from soil and other environments and vectors such as the f-factor based Bacterial Artificial Chromosome (BAC) vectors are used to efficiently clone and propagate large genome fragments. The environmental library approach will process such samples with an aim to archive and replicate with a high degree of fidelity the collective genomes in the mixed microbial assemblage. The basis of this approach is the application of modified Bacterial Artificial Chromosome (BAC) vectors to stably propagate 100-200 kbp genome fragments. The BAC vector and its derivative, the fosmid (for f-factor based cosmid), use the f-origin of replication to maintain copy number at one or two per cell. This feature has been shown to be a crucial factor in maintaining stability of large cloned fragments. High fidelity replication is especially important in propagating libraries comprised of high GC organisms such as the *Streptomyces* from which clones may be prone to rearrangement and deletion of duplicate sequences.

Please replace the original paragraph at page 39, lines 21-28 with the following paragraph:

FACS screening of clones using the methods of the present invention can be performed as described in U.S. Patent Application Number _____, filed June 16, 1997. Other devices which utilize detectors capable of detecting any detectable molecule utilized in a method of the present invention may be employed. Such devices include, but are not limited to, a variety of high throughput cell sorting instruments, robotic instruments, and time-resolved fluorescence

In re Application of:
Jay M. Short
Application No.: 09/529,458
Filed: May 23, 2000
Page 6

PATENT
Attorney Docket No.: DIVER1380-1

instruments, which can actually measure the fluorescence from a single molecule over an elapsed period of time.

Please replace the original paragraph at page 45, lines 8-12 with the following paragraph:
30 milliliters (ml) glass beads (Biospec Products, Bartlesville, OK) are mixed with 50ml
APS/Toluene (10% APS) (Sigma Chemical Co.)
Reflux overnight
Decant and wash 3 times with Toluene
Wash 3 times with ethanol and dry in oven

Please replace the original paragraph at page 45, lines 15-23 with the following paragraph:
25 ml prepared glass beads from above
15 ml 0.1M NaHCO3 + 25 milligrams (mg) N-Acetyl-B-d-glucosamine-PTC (Sigma Chemical Co.) + 1 ml DMSO
Add 10 ml NaHCO3 + 1 ml DMSO
Pour over glass beads
Let shake in Falcon Tube overnight
Wash with 20 ml 0.1M NaHCO3
Wash with 50 ml ddH2O
Dry at 55°C for 1 hour

Please replace the original paragraph at page 46, lines 7-12 with the following paragraph:
Place approximately 1-5mls of the derivitized beads into a Spectra/mesh nylon filter, such as those available from Spectrum (Houston, Texas) with a mesh opening of 70 μ m, an open area of 43%, and a thickness of 70 μ m. Seal the nylon filter to create a "bag" containing the beads using, for instance, Goop, Household Adhesive & Sealant.

In re Application of:

Jay M. Short

Application No.: 09/529,458

Filed: May 23, 2000

Page 7

PATENT

Attorney Docket No.: DIVER1380-1

Please replace the original paragraph at page 47, lines 16-18 with the following paragraph:

A. Digest 10 μ l DNA with EcoRI restriction enzyme (Stratagene Cloning Systems) according to manufacturers protocol electrophoresis DNA digest through 0.5% agarose, 20V overnight; stain gel in 1 μ g/ml EtBr

1. Determine DNA concentration (A_{260} - A_{280}).

Please replace the original paragraph at page 47, lines 22-26 with the following paragraph:

1. Incubate the following at 37°C for 3 hours:
8 μ l DNA (~10 μ g)
35 μ l H₂O
5 μ l 10x restriction enzyme buffer
2 μ l EcoRI restriction enzyme (200 units)

Please replace the original paragraph at page 48, line 4 with the following paragraph:

2. Examine 5 μ l of each fraction on 0.8% agarose gel.

Please replace the original paragraph at page 48, line 9 with the following paragraph:

6. Dry, resuspend in 15 μ l 5T 1E.

Please replace the original paragraph at page 49, lines 3-5 with the following paragraph:

A. Add ~50 nanograms (ng) each of insert and vector DNA to 1U of T4 DNA ligase (Boehringer Mannheim) and 10X ligase buffer as per manufacturers instructions; add H₂O if necessary, to total 10 μ l.

Please replace the original paragraph at page 49, line 19 to page 50, line 10 with the following paragraph:

Cell collection and preparation of DNA. Agarose plugs containing concentrated picoplankton cells were prepared from samples collected on an oceanographic cruise from Newport, Oregon to Honolulu, Hawaii. Seawater (30 liters) was collected in Niskin bottles, screened through 10 μ m Nitex, and concentrated by hollow fiber filtration (Amicon DC10)

In re Application of:

Jay M. Short

Application No.: 09/529,458

Filed: May 23, 2000

Page 8

PATENT
Attorney Docket No.: DIVER1380-1

through 30,000 MW cutoff polysulfone filters. The concentrated bacterioplankton cells were collected on a 0.22 μ m, 47 mm Durapore filter, and resuspended in 1 ml of 2X STE buffer (1M NaCl, 0.1M EDTA, 10 mM Tris, pH 8.0) to a final density of approximately 1×10^{10} cells per ml. The cell suspension was mixed with one volume of 1% molten Seaplaque LMP agarose (FMC) cooled to 40°C, and then immediately drawn into a 1 ml syringe. The syringe was sealed with parafilm and placed on ice for 10 min. The cell-containing agarose plug was extruded into 10 ml of Lysis Buffer (10 mM Tris pH 8.0, 50 mM NaCl, 0.1M EDTA, 1% Sarkosyl, 0.2% sodium deoxycholate, a mg/ml lysozyme) and incubated at 37°C for one hour. The agarose plug was then transferred to 40 mls of ESP Buffer (1% Sarcosyl, 1 mg/ml proteinase-K, in 0.5M EDTA), and incubated at 55°C for 16 hours. The solution was decanted and replaced with fresh ESP Buffer, and incubated at 55°C for an additional hour. The agarose plugs were then placed in 50 mM EDTA and stored at 4°C shipboard for the duration of the oceanographic cruise.

Please replace the original paragraph at page 50, lines 11-26 with the following paragraph:

One slice of an agarose plug seventy-two microliters (72 l) prepared from a sample collected off the Oregon coast was dialyzed overnight at 4°C against 1 ml of buffer A (100 mM NaCl, 10 mM Bis Tris Propane-HCl, 100 μ g/ml acetylated BSA: pH 7.0 @ 25°C) in a 2 mL microcentrifuge tube. The solution was replaced with 250 μ l of fresh buffer A containing 10 mM MgCl₂ and 1 mM DTT and incubated on a rocking platform for 1 hr at room temperature. The solution was then changed to 250 μ l of the same buffer containing 4U of Sau3A1 (NEB), equilibrated to 37°C in a water bath, and then incubated on a rocking platform in a 37°C incubator for 45 min. The plug was transferred to a 1.5 ml microcentrifuge tube and incubated at 68°C for 30 min to inactivate the protein, e.g., enzyme, and to melt the agarose. The agarose was digested and the DNA dephosphorylated using Gelase and HK-phosphatase (Epicentre), respectively, according to the manufacturer's recommendations. Protein was removed by gentle phenol/chloroform extraction and the DNA was ethanol precipitated, pelleted, and then washed with 70% ethanol. This partially digested DNA was resuspended in sterile H₂O to a concentration of 2.5 ng/ μ l for ligation to the pFOS1 vector.

In re Application of:

Jay M. Short

Application No.: 09/529,458

Filed: May 23, 2000

Page 9

PATENT
Attorney Docket No.: DIVER1380-1

Please replace the original paragraph at page 50, line 27 to page 51, line 9 with the following paragraph:

PCR amplification results from several of the agarose plugs (data not shown) indicated the presence of significant amounts of archaeal DNA. Quantitative hybridization experiments using rRNA extracted from one sample, collected at 200 m of depth off the Oregon Coast, indicated that planktonic archaea in this assemblage comprised approximately 4.7% of the total picoplankton biomass (this sample corresponds to "PACI"-200 m in Table 1 of DeLong *et al.*, High abundance of Archaea in Antarctic marine picoplankton, *Nature*, 371:695-698, 1994). Results from archaeal-biased rDNA PCR amplification performed on agarose plug lysates confirmed the presence of relatively large amounts of archaeal DNA in this sample. Agarose plugs prepared from this picoplankton sample were chosen for subsequent fosmid library preparation. Each 1 ml agarose plug from this site contained approximately 7.5×10^5 cells, therefore approximately 5.4×10^4 cells were present in the 72 μ l slice used in the preparation of the partially digested DNA.

Please replace the original paragraph at page 51, lines 10-26 with the following paragraph:

Vector arms are prepared from pFOS1 as described (Kim *et al.*, Stable propagation of cosmid sized human DNA inserts in an f-factor based vector, *Nucl. Acids Res.*, 20:10832-10835, 1992). Briefly, the plasmid is completely digested with *Ast*II, dephosphorylated with HK phosphatase, and then digested with *Bam*HI to generate two arms, each of which contains a *cos* site in the proper orientation for cloning and packaging ligated DNA between 35-45 kbp. The partially digested picoplankton DNA is ligated overnight to the pFOS1 arms in a 15 μ l ligation reaction containing 25 ng each of vector and insert and 1U of T4 DNA ligase (Boehringer Mannheim). The ligated DNA in four microliters of this reaction is *in vitro* packaged using the Gigapack XL packaging system (Stratagene), the fosmid particles transfected to *E. coli* strain DH10B (BRL), and the cells spread onto LB_{cm15} plates. The resultant fosmid clones are picked into 96-well microliter dishes containing LB_{cm15} supplemented with 7% glycerol. Recombinant

In re Application of:

Jay M. Short
Application No.: 09/529,458
Filed: May 23, 2000
Page 10

PATENT
Attorney Docket No.: DIVER1380-1

fosmids, each containing ca. 40 kb of picoplankton DNA insert, have yielded a library of 3,552 fosmid clones, containing approximately 1.4×10^8 base pairs of cloned DNA. All of the clones examined contained inserts ranging from 38 to 42 kbp. This library is stored frozen at -80°C for later analysis.

Please replace the original paragraph at page 52, lines 5-14 with the following paragraph:

Sample composed of genomic DNA is purified on a cesium-chloride gradient. The cesium chloride ($R_f = 1.3980$) solution is filtered through a 0.2 μ m filter and 15 ml is loaded into a 35 ml OptiSeal tube (Beckman). The DNA is added and thoroughly mixed. Ten micrograms of bis-benzimidazole (Sigma; Hoechst 33258) is added and mixed thoroughly. The tube is then filled with the filtered cesium-chloride solution and spun in a VTi50 rotor in a Beckman L8-70 Ultracentrifuge at 33,000 rpm for 72 hours. Following centrifugation, a syringe pump and fractionator (Brandel Model 186) are used to drive the gradient through an ISCO UA-5 UV absorbance detector set to 280 nm. Peaks representing the DNA from the organisms present in an environmental sample are obtained.

Please replace the original paragraph at page 53, lines 4-16 with the following paragraph:

Hybridization screening may be performed on fosmid clones from a library generated according to the protocol described in Example 3 above in any fosmid vector. For instance, the pMF3 vector is a fosmid based vector which can be used for efficient yet stable cloning in *E. coli* and which can be integrated and maintained stably in *Streptomyces coelicolor* or *Streptomyces lividans*. A pMF3 library generated according to the above protocol is first transformed into *E. coli* DH10B cells. Chloramphenicol resistant transformants containing *tcm* or *oxy* are identified by screening the library by colony hybridization using sequences designed from previously published sequences of *oxy* and *tcm* genes. Colony hybridization screening is described in detail in "Molecular Cloning," A Laboratory Manual, Sambrook, *et al.*, (1989) 1.90-1.104. Colonies that test positive by hybridization can be purified and their fosmid clones

In re Application of:
Jay M. Short
Application No.: 09/529,458
Filed: May 23, 2000
Page 11.

PATENT
Attorney Docket No.: DIVER1380-1

analyzed by restriction digestion and PCR to confirm that they contain the complete biosynthetic pathway. (See Figure 6).

Please replace the original paragraph at page 54, lines 2-7 with the following paragraph:

1 μ l fosmid/insert DNA
5 μ l each primer (50 ng/ μ l)
1 μ l Boehringer Mannheim EXPAND Polymerase from their EXPAND kit
1 μ l dNTP's
5 μ l 10X Buffer #3 from Boehringer Mannheim EXPAND kit
30 μ l ddH₂O

Please replace the original paragraph at page 54, line 17 to page 55, line 11 with the following paragraph:

Fosmid DNA from clones that are shown to contain the oxytetracyclin or tetracenomycin polyketide encoding DNA sequences are then used to transform *S. lividans* TK24 *Dact* protoplasts from Example 6. Transformants are selected by overlaying regeneration plates with hygromycin (pMF5). Resistant transformants are screened for bioactivity by overlaying transformation plates with 2ml of nutrient soft agar containing cells of the test organisms *Escherichia coli* or *Bacillus subtilis*. *E. coli* is resistant to the thiostrepton concentration (50 mg/ml) to be used in the overlays of pMF3 clones but is sensitive to oxytetracyclin at a concentration of 5 mg/ml. The *B. subtilis* test strain is rendered resistant to thiostrepton prior to screening by transforming with a thiostrepton marker carried on pHT315. Bioactivity is demonstrated by inhibition of growth of the particular test strain around the *S. lividans* colonies. To confirm bioactivity, presumptive active clones are isolated and cultures extracted using a moderately polar solvent, methanol. Extractions are prepared by addition of methanol in a 1:1 ratio with the clone fermentation broth followed by overnight shaking at 4°C. Cell debris and media solids in the aqueous phase are then separated by centrifugation. Recombinantly expressed compounds are recovered in the solvent phase and may be concentrated or diluted as